Detailed analyses of reductive evolution of virulence-associated genes

Primary virulence genes responsible for invasion of host cells are the virulence gene cluster (*lmo0200-5*) and two internalins (*lmo0433-4*). Among these is the main regulator of virulence *prfA* bearing an N-terminal deletion in 1/2a SLCC5850 and phospholipase *plcA,* necessary for the escape from phagosomes, and showing a premature stop-codon in 3a SLCC7179. Primary virulence genes *inlA* and *inlB* mediate invasion of epithelial cells and a range of other cell types, and display truncations in 3c SLCC2479 and 4b F2365, respectively [1]. Absence of internalin A from the cell wall fraction of strain 3c SLCC2479 was additionally confirmed by immunoblot analysis [2]. These deletions are expected to lead to severe virulence attenuation in the respective strains and were confirmed for strains 1/2a SLCC5850, 3a SLCC7179 and 3c SLCC2479 by low invasion rates of HeLa cells [2]. The deleterious N-terminal mutation of *prfA* in strain SLCC5850 is putatively not responsible for slow growth in BHI medium, as assessed by low degrees of transcription of this gene in this condition, as well as isogenic *prfA* deletion mutants [3]. Instead, growth attenuation of 1/2a SLCC5850 may result from the specific absence of 12 genes found in all other compared strains, coding for various proteins involved in energy production/conversion and metabolism.

One of the repeats of the *actA* gene, which promotes intracellular movement, was previously found to be deleted in the majority of strains of serotype 1/2b and 4b including strains 4b F2365 and 4a L99 [4]. This deletion is also exhibited by strains 3b SLCC2540, 4b L312 and 4e SLCC2378, likely resulting in a limited effect on phenotype.

A range of genes facilitate the attachment to and invasion of host cells. All compared strains of lineage I with the exception of 3b SLCC2540 and 4d ATCC19117 contain the LIPI-3 Listeriolysin S module (*LMOf2365_1113-9*), which translates into a bacteriocin-like haemolytic virulence factor present in strains that caused the majority of epidemics [5]. Strain 7 SLCC2482 shows a premature stop-codon in the bacteriocin biosynthesis cyclohydratase (homologue of *LMOf2365_1117)*. Considering the phylogenetic distribution, LLS was putatively included in an ancestral strain of lineage I and lost in 3b SLCC2540 and 4d ATCC19117 either during separate events or in an ancestral strain of both. Surface proteins Vip and Auto are required for efficient entry into various eukaryotic cell lines [6,7]. The gene encoding Vip is absent from lineage III and all serotype 1/2a strains with the exception of EGD-e, while *aut* could not be found in any serogroup 4 strain, indicating either a reduced range of target cells or a non-homologous replacement in the respective strains. Indeed, all serogroup 4 strains contain a specific amidase gene located at the same relative chromosomal position, which may be able to substitute this functionality. Autolysin Ami can break covalent bonds in the peptidoglycan of cell walls and is involved in binding to host cells by means of GW repeat domains as described for strain EGD-e [8]. Strain 7 SLCC2482 displays a premature stop-codon in the *ami* gene.

As already described, strains of lineage III and especially strain 4a L99 have lost a number of internalins (*inlFC* / *inlFCGHEIJ*), reducing the range of cell types that can be infected, as well as further abilities for adhesion [4,9,10].Interestingly, we identified two distinct versions of *inlJ* in the other strains: variant 1 (2751 bp) is present in lineage I with the exception of 4b L312, variant 2 (~2550 bp) exists in 4b L312 and lineages II/III, respectively. Variant 2 differs from 1 by a central indel of 71

50 bp, resulting in the absence of one of five intestinal mucus binding protein domains
51 (MucBP) [11]. The pattern of distribution of *inlJ* implies the presence of the variant
52 containing four MucBP domains in a *L. monocytogenes* ancestor, followed by
53 duplication of one domain in most strains of lineage I. Taken together, this putatively
54 leads to differing adhesion characteristics of *inlJ* towards eukaryotic cells when
55 comparing the majority of strains of lineage I to II/III.
56 Another class of genes attends to general stress response regulated by alternative
57 sigma factor B (*sigB*) that is itself regulated by multiple other proteins [12-14].
58 Among these are *rsbS, rsbV* and *rsbU,* which contain premature stop-codons in 1/2c
59 SLCC2372, 4d ATCC19117 and 3c SLCC2479, respectively. Affected strains will
60 putatively hold a misregulated *sigB* regulon and thus decreased stress resistance
61 during infection and in the environment. The bile tolerance locus (*lmo0752-4*) and
62 stress survival islet 1 (SSI-1, *lmo0444-8*) are implicated in protecting the bacterium in
63 the gastro-intestinal system and gall bladder [15,16]. Strain 4c SLCC2376 putatively
64 lost the former system including gene *btlB*, while compared strains of serotypes 4a,
65 3a, 3b, 4e, 4d, and 4b do not harbor the latter. All strains do contain homologues of
66 the bile salt hydrolase gene (*bsh*, *lmo2067*) and the bile exclusion locus (*lmo1421-2*)
67 indicating that acid resistance in the deleterious strains is likely diminished but not
68 absent [17]. Furthermore, the complete arginine metabolic pathway (*lmo0036-41*) is
69 absent from lineage III. It is regulated by *sigB* and *prfA,* implied in acid tolerance and
70 described as a virulence factor in the murine model [18].
71 Eukaryotic hosts are described to express a range of cationic antimicrobial peptides
72 that can be countered by bacteria by changing the cell wall composition to include
73 different lipoteichoic acids (eg. *dltA, lmo0974*) [19,20]. *DltA* is part of the regulon of
74 virulence regulator *virR* in *L. monocytogenes* and contains a premature stop-codon in

1/2a SLCC5850, putatively leading to increased sensitivity towards cationic antimicrobial peptides [21].

It was previously demonstrated, that 21 genes were specifically differentially regulated inside IFN-γ-activated macrophages, considered to be the primary host defense effector cells, in comparison to non-activated macrophages [22]. A putatively secreted protein (*lmo0478*) and a protein kinase (*lmo0618*) were not mutually conserved in all compared strains. The former was found to be absent from strain 4a L99 and most strains of lineage I, while the latter shows a C-terminal truncation in strain 1/2b SLCC2755. The functions of these genes have still to be elucidated, but their absence may hamper the ability of the respective strains to survive extreme stresses inside activated macrophages and thus their ability to proliferate inside a host.

MogR (*lmo0674*) was identified in tissue culture models as a motility gene repressor which downregulates *flaA* in the intracellular niche to avoid immune system detection [23]. Strain 3a SLCC7179 displays a premature stop-codon in this gene, putatively leading to increased recognition by the host and thus decreased virulence.

Recently, a study was published describing a secreted virulence factor called LntA (*lmo0438*) targeting the chromatin repressor BAHD1 in the host cell nucleus to activate interferon stimulated genes and thus control bacterial colonization of the host [24]. This gene is absent from serotype 4a (L99 and HCC23) and apathogenic strains of other species being *L. innocua* Clip11262, *L. welshimeri* SLCC5334 and *L. seeligeri* SLCC3954. A nucleotide alignment of *lmo0438* and the corresponding regions in 4a L99 and HCC23 revealed the presence of sequence remnants (stretch of 117 bp with ca. 97% nucleotide identity) indicating a deletion of this gene in an ancestral strain of serotype 4a, putatively contributing to impaired growth of strains of serotype 4a in the host.

Two-component response regulator gene *agrA* (*lmo0051*) was shown to influence the production of several secreted proteins leading to reduced virulence of a deletion mutant in the mouse model [25]. This gene exhibits a premature stop-codon in strain 4e SLCC2378, bearing implications for the virulence of this strain.

The differing availability of nutrients in the environment and in the eukaryotic host necessitates a meta- and catabolic shift for facultative parasitic microbes. Glycerol kinase *glpK2* (*lmo1034*) is a significant member of glycerol catabolism of intracellularly growing *L. monocytogenes* EGD-e in Caco2-cells and was found to be absent from strain 4a L99 [26].

Most compared strains of serotype 1/2a with the exception of EGD-e either showed a low invasion rate of Caco-2 cells (08-5578, 08-5923) or were completely unable to enter this type of cells (SLCC5850) (data not shown). We identified genes related to attachment and invasion that were present in EGD-e and absent from all other compared strains of serotype 1/2a (internalin *lmo1289*, virulence factor *vip*), as well as specific deletions in strains 08-5578/5923 (internalins *lmo0801, lmo2026, lmo2027*), and SLCC5850 (primary virulence regulator *prfA*, phage holin *lmo2279*), that may explain the inability of respective strains to efficiently invade Caco-2 cells. These data indicate that a correlation of Caco-2 invasion rates between strains of the same serotype is not self-evident in support of previous observations [27].

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212

213